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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
10/567,702	08/21/2006	Richard Kim	6750-189-999	3898

20583 7590 12/26/2008
JONES DAY
222 EAST 41ST ST
NEW YORK, NY 10017

EXAMINER	
HOLLERAN, ANNE L	

ART UNIT	PAPER NUMBER
1643	

MAIL DATE	DELIVERY MODE
12/26/2008	PAPER

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Office Action Summary	Application No.	Applicant(s)
	10/567,702	KIM, RICHARD
	Examiner	**Art Unit**
	ANNE L. HOLLERAN	1643

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTH(S) OR THIRTY (30) DAYS, WHICHEVER IS LONGER, FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☒ Responsive to communication(s) filed on 16 September 2008.

2a)☐ This action is **FINAL**. 2b)☒ This action is non-final.

3)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims

4)☒ Claim(s) 2,4,8,10-15,18,22,27,30,34,36 and 47-51 is/are pending in the application.

4a) Of the above claim(s) _____ is/are withdrawn from consideration.

5)☐ Claim(s) _____ is/are allowed.

6)☒ Claim(s) 2, 4, 8, 10-15, 18, 22, 27, 30, 34, 36 and 47-51 is/are rejected.

7)☐ Claim(s) _____ is/are objected to.

8)☐ Claim(s) _____ are subject to restriction and/or election requirement.

Application Papers

9)☐ The specification is objected to by the Examiner.

10)☐ The drawing(s) filed on _____ is/are: a)☐ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

11)☐ The oath or declaration is objected to by the Examiner. Note the attached Office Action or form PTO-152.

Priority under 35 U.S.C. § 119

12)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

a)☐ All b)☐ Some * c)☐ None of:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

* See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☒ Notice of References Cited (PTO-892)
2) ☐ Notice of Draftsperson's Patent Drawing Review (PTO-948)
3) ☒ Information Disclosure Statement(s) (PTO/SB/08)
Paper No(s)/Mail Date 2/07, 12/07, 11/08.
4) ☐ Interview Summary (PTO-413)
Paper No(s)/Mail Date. _____ .
5) ☐ Notice of Informal Patent Application
6) ☐ Other: _____.

DETAILED ACTION

Election/Restrictions

Applicant's election of species, ErbB2 for the first election of species, and "protein" for the second election of species, in the replies filed on 7/22/2008 and 9/16/2008 is acknowledged. Because applicant did not distinctly and specifically point out the supposed errors in the restriction requirement, the election has been treated as an election without traverse (MPEP § 818.03(a)).

In the amendment filed 9/16/2008, claims 47-51 were added. Claims 2, 4, 8, 10-15, 18, 22, 27, 30, 34, 36 and 47-51 are pending and examined on the merits.

Information Disclosure Statement

Several citations in the IDS received 02/05/08 were lined through by the examiner because they are not in conformance with MPEP 609. The lined through citations lack a publication date.

Claim Rejections - 35 USC § 112

The following is a quotation of the second paragraph of 35 U.S.C. 112:

> The specification shall conclude with one or more claims particularly pointing out and distinctly claiming the subject matter which the applicant regards as his invention.

Claims 2, 4, 8, 10-15, 18, 27, and 48 are rejected under 35 U.S.C. 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention.

Claims 2, 27 and 48 are indefinite because of the phrase "therapy regimen for an ErbB-1 positive tumor". It is not clear if this phrase limits the therapy or chemotherapy to a regimen that targets ErbB-1 in particular, or if this phrase is broader in scope and refers to a chemotherapy that is standard for a particular type of cancer, where that particular type of cancer happens to be one that usually expresses ErbB1 (an ErbB-1 positive tumor, such as prostate cancer, for example).

Claim Rejections - 35 USC § 102

The following is a quotation of the appropriate paragraphs of 35 U.S.C. 102 that form the basis for the rejections under this section made in this Office action:

> A person shall be entitled to a patent unless –
>
> (b) the invention was patented or described in a printed publication in this or a foreign country or in public use or on sale in this country, more than one year prior to the date of application for patent in the United States.
>
> (e) the invention was described in (1) an application for patent, published under section 122(b), by another filed in the United States before the invention by the applicant for patent or (2) a patent granted on an application for patent by another filed in the United States before the invention by the applicant for patent, except that an international application filed under the treaty defined in section 351(a) shall have the effects for purposes of this subsection of an application filed in the United States only if the international application designated the United States and was published under Article 21(2) of such treaty in the English language.

Claims 2, 4, 8, 10, 12 13, 15, 22, 30, 34, 36, 47-51 are rejected under 35 U.S.C. 102(b) as being anticipated by Slamon (US 4,968,603; issued Nov 6, 1990).

Slamon teaches a method of measuring the level of ErbB2 in a sample from a patient, such as a patient with beast cancer that has been treated with surgery. Breast cancer is a cancer that also expresses ErbB1. Slamon teaches that higher levels of ErbB2 protein correlate with a more aggressive phenotype, and a shortened time to relapse (see column 2, lines 22- 35). Slamon teaches a method that further comprises treating a subject based on the patient's risk (claims 20 and 21). Slamon teaches

immunohistochemical detection (see column 4, lines 15-42). Thus, Slamon teaches the claimed methods.

Claims 2, 4, 8, 10, 12, 13, 15, 22, 30, 34, 36, 47-51 are rejected under 35 U.S.C. 102(b) as being anticipated by Ross (US 5,994,701; issued Nov 30, 1999).

Ross teaches a method of measuring the level of ErbB2 in a sample from a patient with prostate cancer, which is an ErbB1 positive cancer, where the measurement of ErbB2 is by the detection of ErbB2 protein levels, and the measurement of ErbB2 provides a prognosis (column 3, line 64 – column 4, line 40). Ross teaches a method that comprises a treatment step because Ross teaches that a physician performing therapeutic regimens (see col. 4, lines 35-40). Therefore Ross teaches the claimed inventions.

Claim Rejections - 35 USC § 103

The following is a quotation of 35 U.S.C. 103(a) which forms the basis for all obviousness rejections set forth in this Office action:

> (a) A patent may not be obtained though the invention is not identically disclosed or described as set forth in section 102 of this title, if the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which said subject matter pertains. Patentability shall not be negatived by the manner in which the invention was made.

The factual inquiries set forth in *Graham* v. *John Deere Co.*, 383 U.S. 1, 148 USPQ 459 (1966), that are applied for establishing a background for determining obviousness under 35 U.S.C. 103(a) are summarized as follows:

1. Determining the scope and contents of the prior art.
2. Ascertaining the differences between the prior art and the claims at issue.
3. Resolving the level of ordinary skill in the pertinent art.
4. Considering objective evidence present in the application indicating obviousness or nonobviousness.

Claims 2, 4, 8, 10-13, 15, 22, 30, 34, 36, 47-51 are rejected under 35 U.S.C. 103(a) as being unpatentable over Slamon (supra) as applied to claims 2, 4, 8, 10, 12 ,13, 15, 22, 30, 34, 36, 47-51 above, and further in view of DiGiovanna (DiGiovanna, et al. Cancer Research, 55: 1946-1955, 1995).

The claims encompass a method of detecting ErbB-2 receptor related activity, such as phosphorylation of ErbB-2. Slamon does not teach detection of ErbB2 phosphorylaton.

However, DiGiovanna teaches that measurement of phosphorylation state allows a more accurate test to determine those patients whose tumors are driven by ErbB2 activity and would likely benefit from an anti-ErbB 2 activity. It would have been prima facie obvious to one of ordinary skill in the art at the time the invention was made to have modified the method of Slamon to include a step of measuring ErbB2 receptor related activity, such as phosphorylation level or autophosphorylation activity, which DiGiovanna teaches correlates more closely with a response to therapeutic agents such as an anti-ErbB2 antibody. One would have been motivated by the teaching of DiGiovanna that phosphorylation status of ErbB2 is a more informative predictor of response to treatment using an anti-ErbB2 antibody.

Claims 2, 4, 8, 10, 12, 14, 22, 30, 34, 36, 47-51 are rejected under 35 U.S.C. 103(a) as being unpatentable over Slamon (supra) as applied to claims 2, 4, 8, 10, 12 , 22, 30, 34, 36, 47-51 above, and further in view of Ballinger (US 6,387,638; issued May 14, 2002).

The claims encompass the use of an ErbB receptor ligand as the ErbB receptor probe.

Slamon teaches as set forth above. Slamon fails to teach the use of an ErbB receptor ligand as an ErbB receptor probe. However, Ballinger teaches the use of heregulin variants (see claim 1, column 97-98) to determine whether a sample contains an ErbB receptor. It would have been prima facie obvious to one of ordinary skill in the art at the time the invention was made to have combined the teachings of Slamon with those of Ballinger to make the claimed methods as they relate to use of an ErbB receptor ligand as the ErbB receptor probe. One would have been motivated by the desire to find those tumors expressing ErbB receptors that had ligand binding ability.

Conclusion

No claim is allowed.

Any inquiry concerning this communication or earlier communications from the examiner should be directed to Anne Holleran, whose telephone number is (571) 272-0833. The examiner can normally be reached on Monday through Friday from 9:30 am to 5:00 pm. If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Larry Helms, can be reached on (571) 272-0832. Any inquiry of a general nature or relating to the status of this application or proceeding should be directed to the Group receptionist whose telephone number is (571) 272-1600.

Papers related to this application may be submitted to Group 1600 by facsimile transmission. The faxing of such papers must conform to the notice published in the

Official Gazette, 1096 OG 30 (November 15, 1989). The Official Fax number for Group 1600 is (571) 273-8300.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see http://pair-direct.uspto.gov. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll free).

Anne L. Holleran
Patent Examiner
December 22, 2008
/Alana M. Harris, Ph.D./
Primary Examiner, Art Unit 1643

Notice of References Cited	Application/Control No. 10/567,702	Applicant(s)/Patent Under Reexamination KIM, RICHARD	
	Examiner ANNE L. HOLLERAN	Art Unit 1643	Page 1 of 1

U.S. PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Name	Classification
*	A	US-4,968,603	11-1990	Slamon et al.	435/6
*	B	US-5,994,071	11-1999	Ross et al.	435/6
*	C	US-6,387,638	05-2002	Ballinger et al.	435/7.21
	D	US-			
	E	US-			
	F	US-			
	G	US-			
	H	US-			
	I	US-			
	J	US-			
	K	US-			
	L	US-			
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FOREIGN PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Country	Name	Classification
	N					
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	Q					
	R					
	S					
	T					

NON-PATENT DOCUMENTS

*		Include as applicable: Author, Title Date, Publisher, Edition or Volume, Pertinent Pages)
	U	DiGiovanna, et al. Cancer Research, 55: 1946-1955, 1995
	V	
	W	
	X	

*A copy of this reference is not being furnished with this Office action. (See MPEP § 707.05(a).)
Dates in MM-YYYY format are publication dates. Classifications may be US or foreign.

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Sheet 1 of 3

LIST OF REFERENCES CITED BY APPLICANT (Use several sheets if necessary)	ATTY. DOCKET NO. 6750-189-999	APPLICATION NO. 10/567,702
	APPLICANT Richard Kim	
	U.S. FILING DATE August 21, 2006	ART UNIT 1642

U.S. PATENT DOCUMENTS

*Examiner Initial		Document Number	Date mm/dd/yy	Name Of Patentee Or Applicant Of Cited Document	Pages, Columns, Lines, Where Relevant Passages Or Relevant Figures Appear

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		Foreign Patent Document Country Code, Number, Kind Code (If Known)	Date mm/dd/yy	Name Of Patentee Or Applicant Of Cited Document	Pages, Columns, Lines, Where Relevant Passages Or Relevant Figures Appear	T

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	C01	AARANSON, 1991, "Growth factors and cancer." *Science* 254:1146-53	
	C02	BASELGA et al., 2001, "Mechanism of action of trastuzumab and scientific update." *Semin Oncol.* 28:4-11	
	C03	BASELGA, 2001, "The EGFR as a target for anticancer therapy--focus on cetuximab." *Eur. J. Cancer* 37: S16-22	
	C04	CARRAWAY & CANTLEY, "A neu acquaintance for ErbB3 and ErbB4: a role for receptor heterodimerization in growth signaling." 1994, *Cell* 78:5-8	
	C05	CIARDELLO et al., 2002, "A novel approach in the treatment of cancer: targeting the epidermal growth factor receptor." *Clin. Cancer Res.* 7:2958-70	
	C06	COUSSENS et al., 1985, "Tyrosine kinase receptor with extensive homology to EGF receptor shares chromosomal location with neu oncogene." *Science* 230:1132-9	
	C07	DEBONO et al., 2002, "The ErbB receptor family: a therapeutic target for cancer" Trends in Mol. Med. 8(4): S19-S26	
	C08	FALLS, 2003, :" Neuregulins: functions, forms, and signaling strategies." *Exp. Cell. Res.* 284(1): 14-30	
	C09	FAN et al., 1994, *J. Biol. Chem.* 269:27585-602	
	~~C10~~	~~GENBANK Accession No. AH001455, "Homo sapiens protooncogene protein (c-erb-2) gene, exon 1"~~	
	~~C11~~	~~GENBANK Accession No. AY207002, "Homo sapiens neuregulin 1 isoform 4 (NRG1) mRNA, partial cds,alternatively spliced."~~	
	~~C12~~	~~GENBANK Accession No. NM-004883, "Homo sapiens neuregulin 2 (NRG2), transcript variant 1, mRNA."~~	
	~~C13~~	~~GENBANK Accession No. NM-013981, "Homo sapiens neuregulin 2 (NRG2), transcript variant 2, mRNA."~~	
	~~C14~~	~~GENBANK Accession No. NM-013982, "Homo sapiens neuregulin 2 (NRG2), transcript variant 3, mRNA."~~	
	~~C15~~	~~GENBANK Accession No. NM-013983, "Homo sapiens neuregulin 2 (NRG2), transcript variant 4, mRNA."~~	
	~~C16~~	~~GENBANK Accession No. NM-013984, "Homo sapiens neuregulin 2 (NRG2), transcript variant 5, mRNA"~~	
	~~C17~~	~~GENBANK Accession No. NM-013985, "Homo sapiens neuregulin 2 (NRG2), transcript variant 6, mRNA."~~	
	~~C18~~	~~GENBANK Accession No. NM-031507, "Rattus norvegicus epidermal growth factor receptor (Egfr), mRNA."~~	

EXAMINER /Anne Holleran/ NYI-3927254v1	DATE CONSIDERED 12/22/2008

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	~~C19~~	~~GENBANK Accession No.: AF041792, "Gallus gallus tyrosine kinase ERBB-4 (ERBB4) mRNA, partial cds."~~	
	~~C20~~	~~GENBANK Accession No.: M11762, "Homo sapiens protooncogene protein (c-erb-2) gene, exon 2."~~	
	~~C21~~	~~GENBANK Accession No.: M11763, "Homo sapiens protooncogene protein (c-erb-2) gene, exon 3."~~	
	~~C22~~	~~GENBANK Accession No.: M11764, "Homo sapiens protooncogene protein (c-erb-2) gene, exon 4."~~	
	~~C23~~	~~GENBANK Accession No.: M11765, "Homo sapiens protooncogene protein (c-erb-2) gene, exon 5."~~	
	~~C24~~	~~GENBANK Accession No.: M11766, "Homo sapiens protooncogene protein (c-erb-2) gene, exon 6."~~	
	~~C25~~	~~GENBANK Accession No.: M11767, "Homo sapiens protooncogene protein (c-erb-2) gene, exon 7 and~~ partial cds."	
	C26	GOLDSTEIN et al., 1995, "Biological efficacy of a chimeric antibody to the epidermal growth factor receptor in a human tumor xenograft model." *Clin. Cancer. Res.* 1:1311-8	
	C27	GOUSTIN et al, 1986, "Growth factors and cancer." *Cancer* Res 46: 1015-29	
	C28	HANCOCK et al., 1991, "A monoclonal antibody against the c-ErbB-2 protein enhances the cytotoxicity of cis-diamminedichloroplatinum against human breast and ovarian tumor cell lines." *Cancer Res.* 51:4575-80	
	C29	LOGAN & SHENK, 1984, "Adenovirus tripartite leader sequence enhances translation of mRNAs late after infection." *Proc. Natl. Acad. Sci USA* 81:3655	
	C30	NOONBERG et al., 2000, "Tyrosine kinase inhibitors targeted to the epidermal growth factor receptor subfamily: role as anticancer agents." *Drugs* 59:753-67	
	C31	OLAYIOYE et al., 2000, "The ErbB signaling network: receptor heterodimerization in development and cancer." *EMBO J.* 19:3159-67	
	C32	PLOWMAN et al., 1990, "Molecular cloning and expression of an additional epidermal growth factor receptor-related gene." *Proc. Natl. Acad. Sci USA* 87:4905-9	
	C33	PLOWMAN et al., 1993, "Ligand-specific activation of HER4/p180^{erbB4}, a fourth member of the epidermal growth factor receptor family." *Proc. Natl. Acad. Sci USA* 90:1746-50	
	C34	PREWETT et al., 1996, "The biologic effects of C225, a chimeric monoclonal antibody to the EGFR, on human prostate carcinoma." *J.Immunother. Tumor. Immunol.* 19: 419-427	
	C35	SAMPSON et al., 2000, "Unarmed, tumor-specific monoclonal antibody effectively treats brain tumors." *Proc. Natl. Acad. Sci. USA* 97:7503-8	
	C36	SCHLESSINGER, 2000, "Cell signaling by receptor tyrosine kinases." *Cell* 103:211-225	
	C37	SEDLACEK, 2000, "Kinase inhibitors in cancer therapy: a look ahead." *Drugs* 59:435-76	
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	C40	ULRICH et al., 1984, "Search for selectivity in interactions of chiral solvated electrons." *Nature* 309:418-425	
	C41	WELLS, 1999, "EGF receptor" *Int. J. Biochem & Cell Biol.* 31:637-43	
	C42	WOODBURN, 1999, "The epidermal growth factor receptor and its inhibition in cancer therapy." *Pharmacol. Ther.* 82:241-50	

EXAMINER /Anne Holleran/ NY1-3927254v1	DATE CONSIDERED 12/22/2008

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	U.S. FILING DATE August 21, 2006	ART UNIT 1642

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	C43	YANG et al., 2000, "Therapeutic Potential of ABX-EGF, a Fully Human Anti-EGF Receptor Monoclonal Antibody, for Cancer Treatment." *Proc. Am. Soc. Clin. Oncol.* 19:48	
	C44	ZHANG et al., 1997, "Neuregulin-3 (NRG3): a novel neural tissue-enriched protein that binds and activates ErbB4." *Proc. Natl. Acad Sci USA* 94: 9562-7	

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	APPLICANT Richard Kim	
	U.S. FILING DATE February 2, 2006	ART UNIT ~~1642~~ 1643

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/A.H./	C45	PCT International Search Report dated October 29, 2007, International App. No. PCT/US04/25545.	
/A.H./	C46	NORMANNO, N. et al. "Target-based agents against ErbB receptors and their ligans: a novel approach to cancer treatment." Endocrine-Related Cancer. 2003, Vol. 10, pages 1-21.	
/A.H./	C47	BASELGA, J. et al. Phase I studies of Anti-Epidermal growth factor receptor chimeric antibody c225 alone and in combination with cisplatin. February 2000, Vol. 18, No. 4, pages 904-914.	

EXAMINER /Anne Holleran/	DATE CONSIDERED 12/14/2008

NYI-4046200v1

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	U.S. FILING DATE August 21, 2006	ART UNIT 1643

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[CANCER RESEARCH 55, 1946–1955, May 1, 1995]

Activation State-specific Monoclonal Antibody Detects Tyrosine Phosphorylated p185$^{neu/erbB\text{-}2}$ in a Subset of Human Breast Tumors Overexpressing This Receptor[1]

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ABSTRACT

Amplification of the *neu/erb*B-2/*HER-2* gene and/or overexpression of its receptor tyrosine kinase product, p185$^{neu/erbB\text{-}2}$ (p185), occurs in 15–40% of primary human breast tumors and is variably correlated with poor patient prognosis. Variability in predictive accuracy likely results from activation of p185 by agonist(s) in only a subset of tumors in which it is overexpressed, which may greatly affect outcomes. As a first step toward evaluating this hypothesis, we previously produced a polyclonal antibody that specifically recognizes the activated, tyrosine-phosphorylated forms of p185 and the closely related epidermal growth factor receptor (L. Bangalore *et al.*, Proc. Natl. Acad. Sci. USA, *89*: 11637–11641, 1992). We now describe the production of a mAb, PN2A, that specifically recognizes tyrosine-phosphorylated p185 and bears no cross-reactivity with closely related receptors. Furthermore, we demonstrate its reactivity in immunohistochemical staining of paraffin-embedded, formalin-fixed tumor samples. In a series of five p185-overexpressing human tumors examined thus far, PN2A reactivity was detected in two, indicating that p185 is phosphorylated and hence actively signaling in a subset. This reagent will facilitate both clinical and research analyses of p185 activity. Furthermore, this work serves as a prototype for similar analyses of other tyrosine phosphoproteins.

INTRODUCTION

The *neu/erb*B-2/*HER-2* proto-oncogene encodes a growth factor receptor tyrosine protein kinase (1–5). The *erb*B-2 gene is amplified with resultant overexpression of its protein product, p185$^{neu/erbB\text{-}2}$ (p185), in 15–40% of primary human breast tumors, suggesting a role for this receptor in breast cancer (reviewed in Ref. 6). Numerous experimental approaches substantiate the hypothesis that this alteration contributes significantly to pathogenesis of breast carcinoma. Specific genetic amplification events generally imply selection of a gene conferring a growth advantage to cell clones bearing that alteration. The oncogenically mutated allele of *erb*B-2 is a potent oncogene (7). Unlike other receptor tyrosine kinases including the EGFR,[3] normal p185 is capable of transforming cells in culture when overexpressed even in the absence of ligands (8–10). Infection of rat mammary glands with a retrovirus bearing the oncogenic *neu* gene rapidly induces multifocal mammary carcinomas (11). Similarly, mice transgenic for transforming mutants of p185 develop multifocal mammary carcinoma (12–14). Most significantly, transgenic mice engineered to overexpress structurally normal p185 in mammary tissue develop metastatic mammary carcinoma (15). These animals closely approximate the apparent situation in human breast cancer. Furthermore, overexpression of p185 in human breast tumors has been found in many studies to portend poor prognosis, among both patients with affected lymph nodes (16–18) and node-negative patients (18–20).

p185 is one member of a family of closely related receptors which includes the EGFR, HER-3/*erb*B-3 (21) and HER-4/*erb*B-4 (22). Despite much intensive effort, a ligand that binds to p185 in the absence of other receptors has not yet been unequivocally identified [there are candidates (23)]. However, when p185 is coexpressed with the EGFR, as it often is in cell lines and normal epithelia (reviewed in Ref. 6), EGF (and other EGFR agonists) induces tyrosine phosphorylation of p185 through a process known as transmodulation (24). This is important since both are occasionally co-overexpressed in human breast tumors also (25–27).

Several groups independently identified a family of proteins called HRG/NDF/gp30 as candidate p185 ligands (28–30). However, more recent evidence indicates that they do not bind isolated p185 (31–34). Instead, they are apparently ligands for both HER-3 and HER-4 and can activate p185 via transmodulation (33, 35). Thus, although transactivation has not yet been demonstrated for all of the EGF agonists and HRG/NDF isoforms, transmodulation theoretically could allow regulation of p185 signaling by any EGF agonist acting on the EGFR (EGF, transforming growth factor α, heparin-binding EGF-like growth factor, betacellulin, amphiregulin) and by any HRG/NDF acting through HER-3 and HER-4. Of these, HRGs/NDFs, transforming growth factor α, and amphiregulin are present in mammary tissue and are therefore likely to be important physiological regulators of p185 (reviewed in Refs. 6 and 36).

Early studies of p185 overexpression in human breast tumors demonstrated a correlation with shorter time to relapse and shorter overall survival (16, 17) and multivariate analyses indicated that p185 overexpression has prognostic significance independent of other standard markers. However, the prognostic strength and independence from other markers have been highly variable among follow-up studies, limiting the clinical utility of p185 status (18, 37–45).

Conspicuously, a crucial aspect of the biology of this system has been largely overlooked in the existing studies. These studies have for the most part compared p185 abundance, usually determined by immunohistochemistry, with patient outcome. However, these data do not address the issue of whether the overexpressed receptor is activated by agonists. The overall abundance of receptor tyrosine kinases is not an accurate reflection of their signaling activity for a number of reasons. In the absence of agonists, p185 signaling activity is likely to increase in graded fashion as receptor concentration increases. However, the signaling activity of overexpressed receptors is likely to be radically different in the presence or absence of cognate ligands or transmodulating agonists. Moreover, activation of p185 will induce down-regulation of the receptor, resulting in a lower steady-state receptor abundance. Thus tumors that express receptors most active in signaling will actually display fewer receptors overall than those in which the receptors are overexpressed in the absence of agonists. Finally, although activating structural mutations in p185 have not yet been identified in human tumors, such alterations may in fact exist.

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[3] The abbreviations used are: EGFR, epidermal growth factor receptor; EGF, epidermal growth factor; FBS, fetal bovine serum; HRG, heregulin; PDGF, platelet derived growth factor; NDF, *neu* differentiation factor; pen/strep, penicillin/streptomycin.

and would be capable of imparting a high level of activity to receptors regardless of their level of expression (reviewed in Ref. 6).

For these reasons, determining the signalling status of p185 in breast tumors should have a greater prognostic value than simply determination of its level of expression. One goal of our laboratory has been to devise methodology for assaying the signalling status of p185 directly. This will enable the determination of whether p185 is activated in a distinct subset of p185-overexpressing tumors, particularly those which have been exposed to p185 ligand or physiological transmodulating agonists. Ultimately, this will allow determination of whether these different subsets display differing biological characteristics, particularly with respect to prognosis. By examining the signaling status of p185 directly, the difficulties arising from the lack of knowledge regarding the regulation of p185 activity are circumvented. Additionally, since p185 is under study as a therapeutic target using various antibody treatment strategies, determination of activation of p185 in individual tumors may predict which patients will respond to such immunotherapy.

Our strategy for measuring activation of p185 in tumors is based upon the generalization that tyrosine autophosphorylation marks active forms of receptor tyrosine kinases. Exposure of receptor tyrosine kinases to their cognate ligands induces their tyrosine phosphorylation through the action of their intrinsic catalytic domains. Using antiphosphotyrosine immunoblotting, it has been shown previously that overexpressed p185 is tyrosine phosphorylated in breast cell lines (46) and also in human breast tumors (47).[4] Antibodies that recognize phosphotyrosine in the context of a specific peptide sequence permit exploitation of tyrosine phosphorylation as an indicator of signaling activity (48, 49). We previously reported production of a polyclonal antibody that specifically recognizes the autophosphorylated (*i.e.*, signaling-active) form of p185. This antibody was produced by immunizing rabbits with a phosphotyrosine-containing synthetic peptide corresponding to the COOH-terminal p185 autophosphorylation site (48). The antibody preferentially recognized phosphorylated p185 but also recognized phosphorylated EGFR (48), which is highly homologous in the region of this autophosphorylation site. We now report the production of a phosphorylation state specific anti-p185 mAb bearing no cross-reactivity with other members of the EGFR family. This antibody reacts with only a subset of p185-overexpressing tumors, supporting our hypothesis that the signaling activity of p185 varies among different tumors.

MATERIALS AND METHODS

Cell Culture. SKBR3 cells are a human mammary carcinoma cell line that overexpresses p185. A431 cells express high levels of the EGFR and moderate levels of p185. NR6 cells express substantial levels of PDGF receptor. J4 cells and N54 cells are derivatives of NIH3T3 murine fibroblasts that were transformed by transfection with v-*src* and v-*abl* oncogenes, respectively. ψ2/HER-4 cells are a derivative of ψ2 cells that have been transfected with an LXSN retroviral expression vector containing a cDNA insert encoding HER-4.[5]

SKBR3 cells were cultured in McCoy's 5A modified medium, supplemented with 15% heat-inactivated FBS, glutamine, and pen/strep. A431 cells, NR6 cells, NIH3T3 cells, and their derivatives were cultured in DMEM, supplemented with 10% heat-inactivated FBS and pen/strep. ψ2/HER-4 cells were cultured in the same medium with the addition of 300 μg/ml G418. Hybridoma cells were grown in RPMI, supplemented with 10% heat-inactivated FBS, glutamine, pen/strep, and HEPES.

Reagents. Anti-p185 mAb Ab-3 (clone 3B5) and polyclonal antibody Ab-1 were purchased from Oncogene Science, Inc. (Manhasset, NY). Anti-EGFR mAb 528 (50) was a gift from H. Masui (Rockefeller University, New York, NY). Polyclonal anti-phosphotyrosine antibody was produced as described in Ref. 51. EGF and mouse nonspecific IgG1 were purchased from Sigma (St. Louis, MO). A synthetic peptide corresponding to amino acid residues 177–241 of HRGβ1 was a gift from James Moyer (Pfizer Central Research, Groton, CT). Recombinant human PDGF-BB, a gift from Daniel DiMaio, was purchased from Collaborative Biomedical Products (Bedford, MA). Polyclonal anti-HER-4 antibody (*erb*-B4 C-18) was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz CA). The Sigma Fast 3,3′-diaminobenzidine staining kit was purchased from Sigma.

Production of Phosphorylation State-specific mAb. The phosphotyrosine (pY)-containing peptide KTAENPE-pY-LGLDVPV, which corresponds to the COOH-terminal 14 amino acids of p185 plus an NH_2-terminal lysine for coupling ("phospho-*neu* peptide"), and the identical non-phosphate-containing peptide ("non-phospho-*neu* peptide") were synthesized by James Eliot (W. M. Keck Foundation Biotechnology Resource Laboratory, Yale University) as described previously (48). The tyrosine residue in this sequence corresponds to the COOH-terminal-most major autophosphorylation site in p185 (52). The pY-containing peptides KTAENAE-pY-LRVAPQS, corresponding to the homologous COOH-terminal EGFR autophosphorylation site, and RLIEDNE-pY-TARQGAK, corresponding to the major c-*src* autophosphorylation site surrounding Tyr 416 within the kinase domain, were synthesized in like manner. The pY-containing peptide TSTEPQ-pY-QPGENL, corresponding to the c-*src* autophosphorylation site surrounding Tyr 527, was a gift from Dr. Joan Levy (Yale University).

Phospho-*neu* peptide was coupled to keyhole limpet hemocyanin with glutaraldehyde. Five female BALB/c mice were immunized with a 50% emulsion of 1 mg/ml conjugate in complete Freund's adjuvant on day 1 and boosted on days 15 and 37 with the conjugate in incomplete Freund's reagent. Test bleeds taken on day 47 were analyzed for phospho-*neu* titer by ELISA. On day 57 the mouse with the best titer was boosted i.v., and the spleen was harvested on day 60. Fusion of splenocytes, selection, and propagation of hybridomas was performed by Jane Dunn and Dr. Kim Bottomly at the Yale Medical School/Howard Hughes Memorial Institute hybridoma facility using standard techniques. Of approximately 1200 single cell colony culture medium supernatants from a single fusion screened by ELISA for phospho-*neu* peptide reactivity, 68 were positive. Twenty of these lacked ELISA cross-reactivity with the non-phospho-*neu* peptide, and 13 of these 20 displayed no cross-reactivity with the Tyr 416 phospho-*src* peptide (indicating that they were not merely anti-pY antibodies). Three of these 13 exhibited slight cross-reactivity with the highly homologous phospho-EGFR peptide. The remaining 10 hybridomas were subcloned by limiting dilution, after which only 5 continued to produce anti-phospho-*neu* antibody. Of these, one clone, designated PN2A ("phospho-*neu* clone 2A"), detected phosphorylated p185 reliably in immunoprecipitation (data not shown) and immunoblotting assays (see below). Isotyping using the ImmunoPure mAb isotyping kit (Pierce, Rockford, IL) revealed this antibody to be of the IgG1κ class.

For large scale production of PN2A, the hybridoma cells were inoculated i.p. into pristane-primed BALB/c mice for production of ascites fluid. Ascites fluid was partially purified by ammonium sulfate precipitation and dialyzed against PBS (137 mM NaCl-8 mM Na_2HPO_4-2.6 mM KCl-1.5 mM KH_2PO_4, pH 7.3) containing 0.01% azide. The dialyzed product was clarified and filter sterilized through a 0.22 μm membrane. This preparation was used without further purification.

ELISA Screening. Vinyl assay plates (96 wells; Costar, Cambridge, MA) were coated with a 10 μg/ml solution of BSA-conjugated peptides in 75 mM NaCl-100 mM boric acid-47 mM sodium borate (pH 8.4), blocked with 1% BSA in 75 mM NaCl-100 mM boric acid-47 mM sodium borate (pH 8.4)/0.01% azide, and incubated with sample antibody diluted in blocking solution. Plates were subsequently incubated with biotinylated horse anti-mouse antibody (Vector Laboratories, Burlingame, CA) followed by horseradish peroxidase-conjugated avidin D (Vector). The substrate used was 0.5 mg/ml 4-aminoantipyrene in 0.17 M phenol/7 $\times$ 10^{-3}% H_2O_2. Wells were scored positive (deep red color) or negative (absence of color).

Immunoprecipitations. Confluent SKBR3 cells in 100-mm dishes were serum starved overnight in medium containing 0.1% FBS. To maximize differential phosphorylation of p185 isolated from EGF- and mock-treated cells, the phosphotyrosine phosphatase inhibitor sodium o-vanadate was added at a final concentration of 500 μM during the final h prior to stimulation of

[4] Unpublished data.

[5] D. J. Riese II and D. F. Stern, manuscript in preparation.

cultures that were to be stimulated with EGF and was present at all subsequent steps pre- and postlysis. Mock-treated cells were incubated pre- and postlysis in solutions lacking sodium *o*-vanadate to allow cellular phosphotyrosine phosphatases the opportunity to reverse any basal phosphorylation. Cells were incubated with EGF at 100 ng/ml for 7 min at room temperature with sodium *o*-vanadate or mock-incubated in the absence of EGF. Cells were immediately washed on ice and solubilized in 1.5 ml of lysis buffer consisting of 10 mM sodium phosphate (pH 7.2), 150 mM NaCl, 1 mM EDTA, 1% NP40, and 1% aprotinin, with or without freshly added 1 mM sodium *o*-vanadate. Five μg of anti-p185 Ab-1 were used to immunoprecipitate p185 from 1 ml of lysate. EGFR was immunoprecipitated from A431 cells in similar fashion, except that the lysis buffer used was 10 mM sodium phosphate (pH 7.0), 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 1% sodium deoxycholate, and 1% aprotinin with or without 1 mM sodium *o*-vanadate. EGFR immunoprecipitation was performed using 4 μl of antibody 528 followed by 2 μg of goat anti-mouse antibody 31160 (Pierce). HER-4 was immunoprecipitated from confluent ψ2/HER-4 cells in 100-mm dishes in a manner similar to that for p185, except that the stimulated plates were incubated with 50 ng/ml of synthetic HRGβ1 peptide in addition to EGF and *o*-vanadate. Five μg of anti-HER-4 antibody were used to immunoprecipitate HER-4 from 1 ml of lysate. Immune complexes were collected with protein A-Sepharose CL-4B (Pharmacia, Uppsala, Sweden).

Whole Cell Solubilization. Growth factor stimulated cells were treated as for immunoprecipitations. Cells were subsequently washed and then solubilized in 2 ml electrophoresis sample buffer at 100°C.

Immunoblotting. Samples were resolved in 7.5% acrylamide/0.175% bis-SDS-polyacrylamide electrophoretic gels at constant current followed by transfer to Hybond-ECL nitrocellulose and processing using enhanced chemiluminescence (Amersham, Arlington Heights, IL). Anti-p185 Ab-1 was used at a concentration of 1 μg/ml and PN2A was used at a 1:1000 dilution. The secondary peroxidase-labeled antibodies were used at 1:10,000 dilution. For anti-HER-4 blotting, primary antibody was used at 1 μg/ml and secondary at 1:1000 dilution.

Immunofluorescence. Indirect immunofluorescence was performed as described (48). PN2A was used at a dilution of 1:500 and was detected with biotinylated goat anti-mouse antibody (Vector) followed by rhodamine-conjugated avidin (Vector).

Immunohistochemistry. Surgical specimens were fixed in 10% neutral buffered formalin and paraffin embedded by standard procedures. Immunohistochemistry was performed based on the peroxidase anti-peroxidase method of Press *et al.* (53). Four micron sections were deparaffinized and rehydrated through graded alcohols, quenched in 0.5% H_2O_2 in PBS for 15 min, and rinsed twice with PBS. Blocking was performed using 10% normal rabbit serum in PBS for 20 min at room temperature, and primary antibodies were applied overnight at 4°C in a humid chamber. All antibodies were diluted in 10% normal rabbit serum/PBS and precleared by incubation with Rheumatex latex reagent (Wampole Laboratories, Cranbury, NJ), 50 μl/ml of antibody solution, rotating at 4°C for a minimum of 2 h. Anti-p185 Ab-3 was used at 0.02 μg/ml, and PN2A was used at 1:100 dilution. Samples were then washed with PBS and incubated with secondary bridging rabbit anti-mouse antibody (Zymed, South San Francisco, CA) at 1:50 dilution for 30 min at room temperature, followed by washing and incubation with mouse peroxidase anti-peroxidase (Sternberger Monoclonals, Inc., Baltimore, MD) at 1:50 dilution for 30 min at room temperature. For PN2A, as well as nonspecific IgG1 control, application of secondary and tertiary antibodies was repeated a second time to enhance sensitivity. The reaction was developed using the Sigma Fast 3,3'-diaminobenzidine staining kit and counterstained with ethyl green (Cell Analysis Systems, Inc., Elmhurst, IL). For experiments using blocking peptides, primary antibody was preincubated with 0.1 volume of a 4-mg/ml solution of peptide for 15 min on ice prior to application to the section.

RESULTS

Production of PN2A. We previously produced a polyclonal phosphorylation state-specific anti-p185 antibody (48). This antibody is highly sensitive but requires some effort to produce owing to the many affinity purification steps, is subject to differences in specificity depending on the immune response of the individual rabbit immunized, and cross-reacts with the EGFR. We therefore sought to produce analogous p185-specific phosphorylation-dependent mAbs to circumvent these problems. Mice were immunized with the phospho-*neu* peptide, and hybridoma conditioned media were screened consecutively for reactivity with this peptide, but failure to react with non-phospho-*neu*, Tyr 416 phospho-*src* or phospho-EGFR peptides as described in "Materials and Methods." The most promising mAb, PN2A, immunoprecipitated solubilized phosphorylated p185 (data not shown), demonstrating its ability to recognize the intact native protein as well as the peptide used in ELISA screens.

Specificity of PN2A. We first determined whether PN2A reacts with p185 in a phosphorylation-dependent manner. These experiments

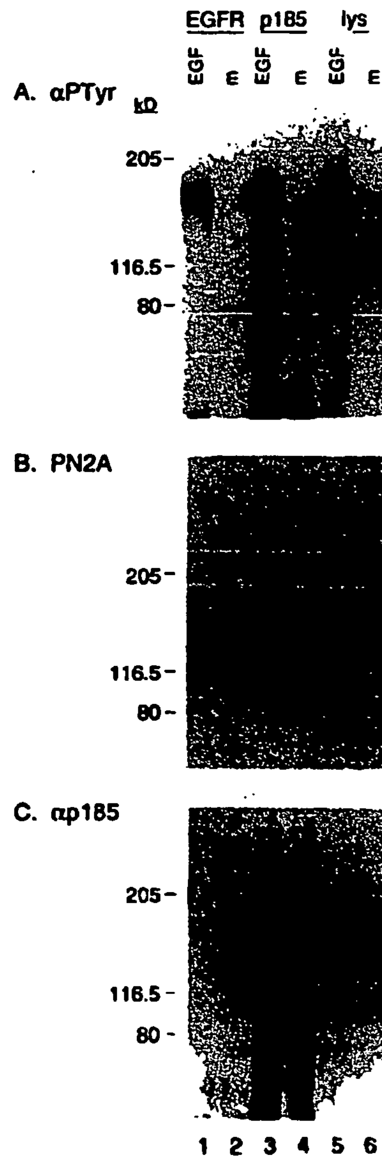

Fig. 1. Immunoblot analysis of p185 and EGFR with PN2A. Immunoblot analyses of EGFR immunoprecipitates (*Lanes 1* and *2*), p185 immunoprecipitates (*Lanes 3* and *4*), and SKBR3 whole cell lysates (*lys*; *Lanes 5* and *6*) with anti-phosphotyrosine (*A*), PN2A (*B*), and anti-p185 Ab-1 (*C*). EGFR was immunoprecipitated from A431 cells and p185 was immunoprecipitated from SKBR3 cells. Prior to cell lysis, cells received EGF (*Lanes 1, 3,* and *5*) or mock (*m*; *Lanes 2, 4,* and *6*) stimulation. (In Figs. 1 and 3, the p185 band in *B* appears larger relative to the molecular weight markers than it does in *A*. This artifact was due to "smiling" of the gel from which these blots were taken.) *kD*, molecular weight in thousands.

were performed in SKBR3 cells, a human mammary carcinoma line that expresses high levels of p185. p185 phosphorylation can be monitored after immunoprecipitation of p185 (Fig. 1, *Lanes 3* and *4*) or in total cellular protein extracts (*Lanes 5* and *6*). These cells also express the EGFR, permitting regulation of p185 tyrosine phosphorylation through transmodulation with EGF (48). Transient treatment of SKBR3 cells with EGF has no effect on p185 levels detected by immunoblotting with anti-p185 (Fig. 1*C*, *Lanes 3–6*). However, EGF greatly stimulated tyrosine phosphorylation of p185, as determined by immunoblotting with anti-phosphotyrosine (Fig. 1*A*, *Lanes 3–6*). The intensity of p185 detected by immunoblotting with PN2A displayed a similar phosphorylation dependence (Fig. 1*B*, *Lanes 3–6*). Thus PN2A recognizes p185 in a phosphorylation-dependent manner.

The polyclonal anti-phospho-*neu* antibody that we produced previously cross-reacted with the closely related EGFR (48), which is 50% identical to p185 at the site of the peptide immunogen (see "Materials and Methods" for peptide sequences). To verify the ability of PN2A to discriminate between p185 and the EGFR, immunoblotting was performed on immunoprecipitated p185 and EGFR and on whole cell lysates of SKBR3 cells (Fig. 1). As discussed above, tyrosine phosphorylation of both receptors is dramatically stimulated by EGF (Fig. 1*A*, *Lanes 1–4*) in the absence of changes in abundance of p185 (Fig. 1*C*, *Lanes 3* and *4*). PN2A recognized p185 in a tyrosine phosphorylation-dependent manner (compare Fig. 1*B*, *Lanes 3–6* (PN2A), to Fig. 1*A*, *Lanes 3–6* (anti-phosphotyrosine)). However, in contrast to anti-phosphotyrosine, PN2A failed to recognize the tyrosine-phosphorylated EGFR (Fig. 1, *A* and *B*, *Lane 1*).

The COOH terminus of HER-4, like the EGFR, encompasses a peptide highly homologous to p185. Hence we used a similar strategy to evaluate cross-reactivity of PN2A with HER-4 (Fig. 2). The M_r 180,000 protein detected by consecutive immunoprecipitation and immunoblotting with anti-HER-4 (Fig. 2*C*, *Lanes 2* and *3*) is specific to clones of ψ2 cells transfected with a HER-4 cDNA expression vector.[6] Treatment of ψ2/HER-4 cells with a cocktail of HRGβ1 peptide, EGF and *o*-vanadate stimulated tyrosine phosphorylation of the HER-4 protein (Fig. 2*A*, *Lanes 2* and *3*). However, PN2A immunoblotting of these samples failed to detect phosphorylated HER-4, although the intensity of the p185 signal in lysates from SKBR3 cells was equivalent with anti-phosphotyrosine and PN2A (Fig. 2, *A* and *B*). [The lower molecular weight bands detected in the HER-4 and phosphotyrosine immunoblots are likely proteolytic fragments of HER-4, since the bands from stimulated cells all consistently migrate with a slight mobility shift compared to unstimulated cells (Fig. 2*C*, *Lanes 2* and *3*), presumably due to the addition of phosphate. Furthermore, these bands are absent from anti-HER-4 immunoblots of ψ2 cells transfected with empty vector.[6]] Taken together, these data demonstrate that PN2A specifically recognizes p185, but not the closely related EGFR or HER-4, in a phosphorylation-dependent manner. A homologous peptide is not present in the remaining member of this receptor family, HER-3. Hence, among the EGFR family of receptors, PN2A exhibits strict specificity for phosphorylated p185.

To further demonstrate the ability of PN2A to discriminate phosphorylated p185 from other tyrosine-phosphorylated proteins, immunoblotting was performed on total cell protein extracts of a series of cell lines activated with growth factors or oncogenic tyrosine protein kinases (Fig. 3). Anti-phosphotyrosine blotting revealed a wide variety of phosphotyrosyl proteins in these lysates (panel A). They include p185 in EGF-stimulated SKBR3 cells (Fig. 3*A*, *Lane 1*), the PDGF receptor in mock- or PDGF-stimulated NR6 cells (Fig. 3*A*, *Lanes 2* and *3*), and numerous substrates in v-*src*- and v-*abl*-transformed J4

[6] D. J. Riese II and D. F. Stern, unpublished observations.

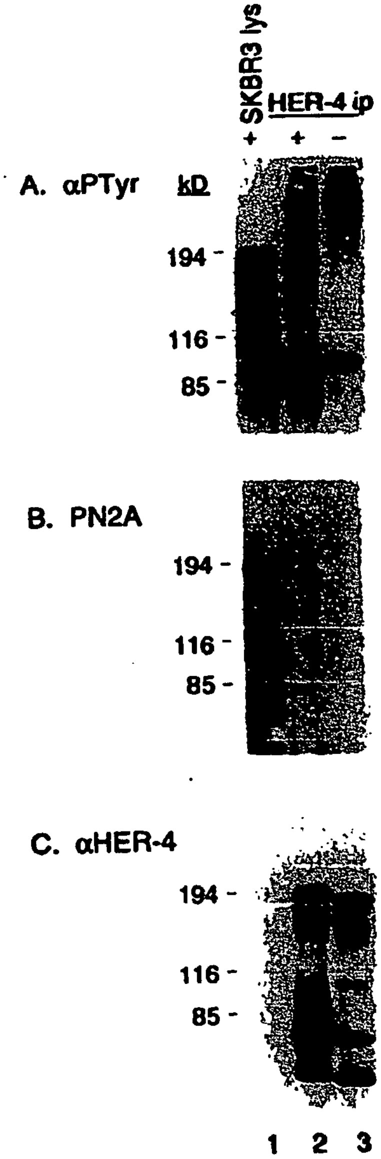

Fig. 2. Immunoblot analysis of HER-4 with PN2A. Immunoblot analyses of HER-4 immunoprecipitates (*Lanes 2* and *3*) and SKBR3 whole cell lysate (*Lane 1*) with anti-phosphotyrosine (*A*), PN2A (*B*), and anti-HER-4 antibody (*C*). HER-4 was immunoprecipitated from ψ2/HER-4 cells which were stimulated (+; *Lane 2*) or mock treated (−; *Lane 3*) with a cocktail of HRGβ1 peptide, EGF, and *o*-vanadate. SKBR3 cells were treated with EGF prior to lysis. *kD*, molecular weight in thousands.

(Fig. 3*A*, *Lane 5*) and N54 (Fig. 3*A*, *Lane 6*) cells. As expected, few bands were evident in immunoblots of nontransformed NIH3T3 cells (Fig. 3*A*, *Lane 4*). However, the sole phosphotyrosine-containing protein detected by PN2A was p185 from SKBR3 cells (Fig. 3*B*, *Lane 1*).

Immunofluorescence of SKBR3 Cells. Since the most important applications for this antibody will be in cell-based assays, we determined whether PN2A will detect cell-associated phosphorylated p185 (Fig. 4). SKBR3 cells were stimulated with EGF and analyzed by indirect immunofluorescence. EGF treatment greatly enhanced fluorescence of these cells (compare Fig. 4*B* and Fig. 4*F*). This signal was blocked by preincubation of PN2A with phospho-*neu* peptide (*neu* Ppep; Fig. 4, *C* and *D*) but was unaffected by the non-phospho-*neu*

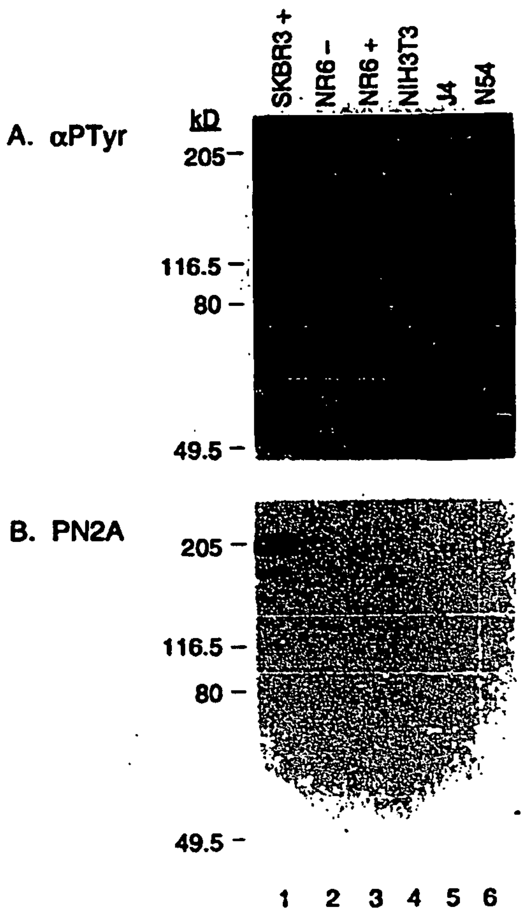

Fig. 3. Specificity of PN2A. Immunoblot analyses of whole cell lysates of EGF-treated SKBR3 cells (*Lane 1*), mock-treated (*Lane 2*), or PDGF-BB-treated (*Lane 3*) NR6 cells, NIH3T3 cells (*Lane 4*), J4 cells (*Lane 5*), and N54 cells (*Lane 6*). Membranes were probed with anti-phosphotyrosine antibody (*A*) and PN2A (*B*). *kD*, molecular weight in thousands.

blocking peptide (neu pep; Fig. 4, *A-B* and *E-F*), confirming specificity of staining. Thus, cell-associated p185 is recognized by PN2A in a specific and EGF-regulated fashion.

Immunostaining of Human Breast Tumors. Immunohistochemical staining with PN2A was performed on a specimen of formalin-fixed, paraffin-embedded human breast tumor (Fig. 5). Immunostaining with anti-p185 antibody Ab-3, which recognizes the COOH-terminal peptide but is insensitive to phosphorylation state, revealed intense membranous immunostaining (Fig. 5*B*). No staining was observed with nonspecific IgG1 (Fig. 5*D*). Immunostaining with PN2A displayed a pattern similar to that seen for Ab-3, but with somewhat reduced intensity and less uniformity among all neoplastic cells (Fig. 5*C*). In addition to strong membranous staining, PN2A showed very light cytoplasmic staining. The strong membranous staining, but not the faint cytoplasmic staining, was blocked by the phospho-*neu* peptide (Fig. 5F), but not by the non-phospho-*neu* peptide (Fig. 5*E*), the Tyr 527 phospho-*src* peptide (Fig. 5*G*), or the phospho-EGFR peptide (Fig. 5*H*). These blocking experiments establish the specificity of the membrane-associated PN2A staining. Thus, PN2A specifically recognizes tyrosine-phosphorylated p185 in formalin-fixed, paraffin-embedded specimens.

As discussed above, we believe that the biological activity of p185 in human tumors may vary greatly, depending upon the constellation of agonists to which it is exposed. To test the prediction that p185 is actively signaling in only a subset of the tumors in which it is overexpressed, we screened a panel of paraffin-embedded sections of mammary tumors with Ab-3 for overexpression of p185. Four additional p185-overexpressing tumors were tested for PN2A reactivity (Fig. 6). All four tumors showed strong membranous immunostaining with Ab-3 (Fig. 6, *B, E, H,* and *K*), but only one displayed robust membranous immunostaining with PN2A (Fig. 6*C*). Thus, in this series of five tumors that overexpress p185, only two appear to express activated, phosphorylated receptor. Hence, these data support the notion that only a subset of p185-overexpressing tumors possess activated receptor.

DISCUSSION

We have produced a mAb, PN2A, that is specific to the Tyr-1248 phosphorylated p185 product of the *neu/erb*B-2/HER-2 proto-oncogene. The stringent specificity of PN2A for tyrosine-phosphorylated p185 and lack of cross-reactivity with a broad range of phosphotyrosyl proteins, including the closely related HER-4 and EGFR, has been verified using a series of immunoblotting and peptide blocking experiments. Since this reagent can be used to stain paraffin-embedded tissue sections, it will allow direct analysis of the signaling status of p185 in cellular material. This will circumvent the difficulty in predicting p185 activity arising from the complexity of known and possible unknown agonists for p185.

Direct staining of intact tissue for specific activated receptors using previous techniques was impossible. Conventional anti-phosphotyrosine antibodies detect not only the activated receptor of interest but also all cellular phosphotyrosine-containing proteins. Staining with conventional anti-receptor-specific antibody suffers from the inability to discriminate activated from unactivated receptor. Quantification of receptor phosphorylation in tumor samples has traditionally required multiple steps including extraction, immunoprecipitation of receptors, and then immunoblotting with anti-phosphotyrosine (47). Routine clinical screening by such methodology is impractical. Additionally, such experiments are subject to inaccurate interpretations because of variability in tissue content of stroma/normal cells *versus* tumor cells and because of tumor cell heterogeneity (38). Moreover, the biological information contained in the histological detail is lost. PN2A staining circumvents these obstacles.

The interpretation of p185 immunohistochemical staining patterns has generated a fair amount of controversy in the literature. While many breast tumor samples display plasma membrane staining, as would be expected for an integral membrane receptor, some tumors as well as other tissues display granular cytoplasmic staining (53–55). In at least some cases, cytoplasmic staining appears to be associated with mitochondria, and anti-p185 immunoblotting of extracts from tumors with this pattern detects a M_r 155,000 protein. Press *et al.* (53) have demonstrated that membranous staining, but not cytoplasmic staining, correlates well with results obtained by Southern, Northern, and immunoblotting. One study of 888 node-positive patients demonstrated correlation of membranous staining with poor prognosis, whereas cytoplasmic staining did not correlate with prognosis (56). In another study of 463 patients, cytoplasmic staining actually correlated with favorable prognostic factors (57). In this study, while membranous staining correlated with decreased survival, there was no survival difference among cases with absence of staining, cytoplasmic staining, or even the combination of membranous and cytoplasmic staining.

Bona fide cytoplasmic p185 staining should occur to some extent when p185 is activated since this leads to internalization of the receptor (58). Treatment of breast carcinoma cells with differentiation-inducing agents leads to progressive loss of p185 surface mem-

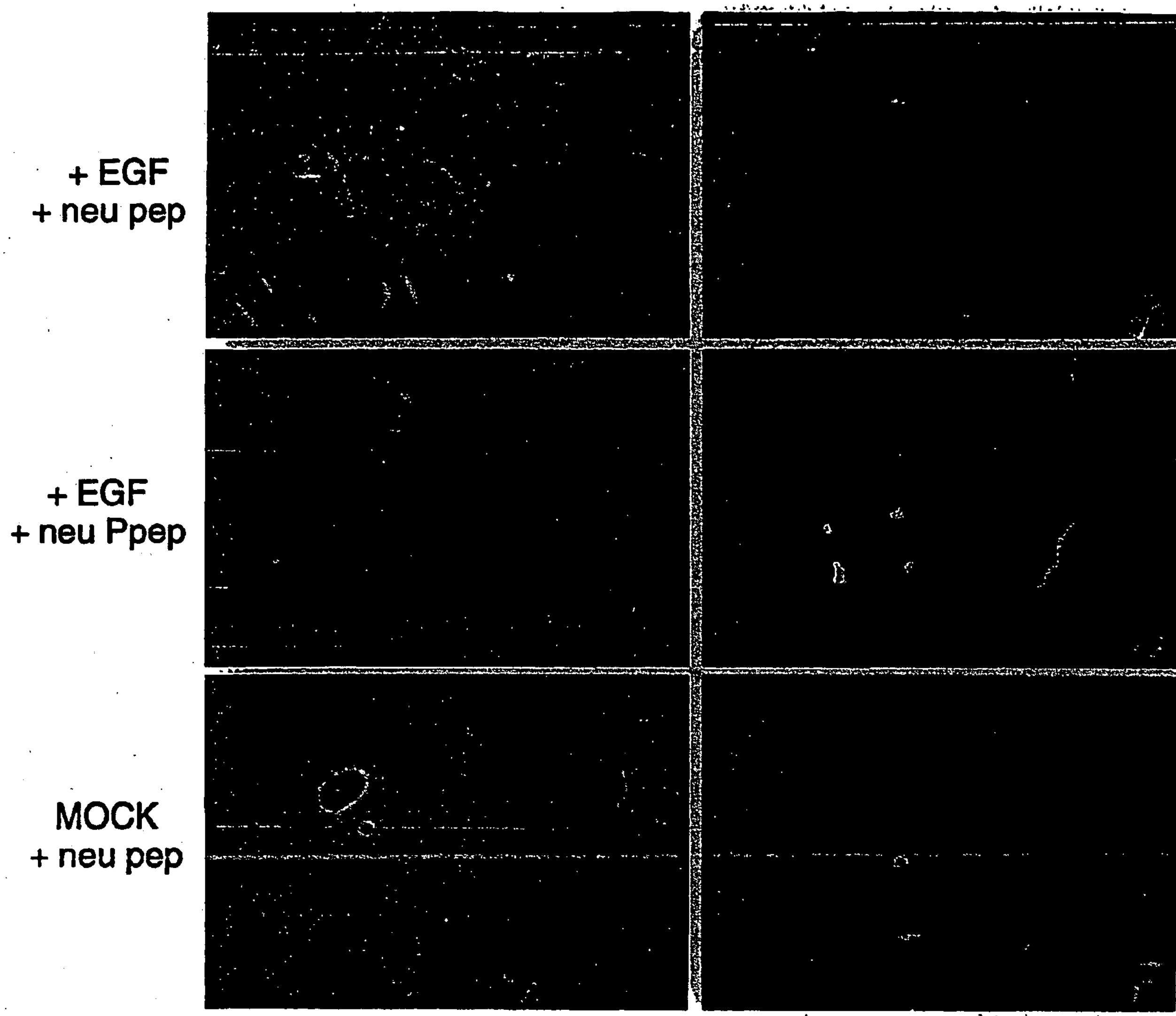

Fig. 4. Indirect immunofluorescence of SKBR3 cells using PN2A. Cells were pretreated with EGF (*A-D*) or mock stimulated (*E-F*). PN2A was preincubated with non-phospho-*neu* peptide (*neu pep*; *A, B, E, F*) or phospho-*neu* peptide (*neu Ppep*; *C* and *D*). Staining was detected with biotinylated goat anti-mouse antibody followed by rhodamine-conjugated avidin. Cells were photographed under phase contrast (*A, C, E*) or immunofluorescence (*B, D, F*) microscopy. Each pair represents one microscopic field.

brane reactivity accompanied by transiently increased cytoplasmic staining (59). Tyrosine phosphorylation of p185 not only marks signaling receptor, but, based upon precedent in the EGFR system, probably targets the receptor for endocytosis (60, 61). Thus, PN2A likely identifies the subset of molecules destined for internalization. However, the results of the peptide blocking experiments in Fig. 5 suggest that only the strong membranous staining and not the weak cytoplasmic staining is specific, since the phospho-*neu* peptide failed to block the latter. Nevertheless, in staining further specimens we have observed that while nearly all show some faint cytoplasmic staining, this is sometimes decreased or even eliminated with the phospho-*neu* peptide block.[4] Hence, the interpretation of cytoplasmic staining remains a point of contention.

The staining pattern of PN2A observed thus far has also been heterogeneous among the tumor cell population within a given specimen, despite uniformity of staining with anti-p185 Ab-3, and also is consistently less intense than with anti-p185 Ab-3. There are a number of possible reasons for this: (*a*) the stoichiometry of phosphorylation of p185 may be intermediate or low; (*b*) the affinity of PN2A may be considerably less than that of Ab-3; (*c*) phosphotyrosine may not be stable to fixation and paraffin embedding.

We have evidence that the third possibility is not the case. We have prepared formalin-fixed, paraffin-embedded cell blocks of EGF-treated SKBR3 cells and we find that we are able to stain these cells with PN2A.[4] Studies by other investigators have demonstrated immunostaining with monoclonal anti-phosphotyrosine antibodies in paraffin-embedded human breast biopsy tumor samples (62). Of the two PN2A-positive tumors shown in the present work, one was accessioned in 1994 (Fig. 5) and one in 1989 (Fig. 6, *A-C*), indicating stability over a period of years. In fact, the tyrosine-phosphate linkage is known to be chemically very stable to wide extremes of conditions. The heterogeneous staining pattern with PN2A may be due to activation of p185 in specific compartments within a given tumor, *e.g.*, in regions where the receptor has access to an activating ligand.

The exact sequence by which p185 promotes human mammary tumorigenesis is open to speculation, but some clues exist. Overex-

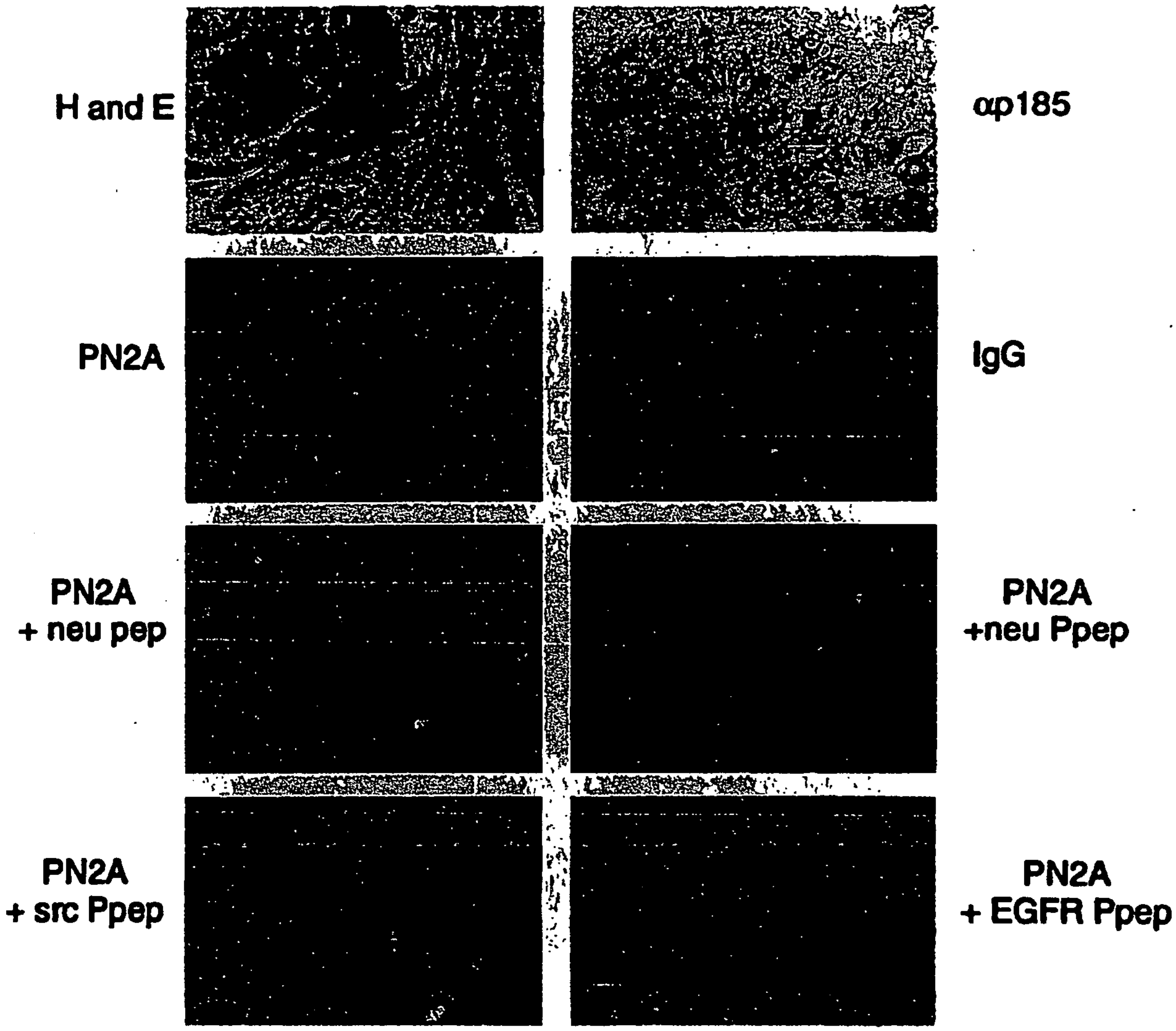

Fig. 5. Specificity of PN2A in immunohistochemistry. Immunohistochemical staining of a human breast tumor with anti-p185 antibody Ab-3 (*B*), nonspecific IgG1 (*D*) and PN2A (*C, E-H*). H & E staining is also shown (*A*). PN2A was preincubated in the presence of non-phospho-*neu* peptide (*E*), phospho-*neu* peptide (*F*), Tyr 527 phospho-*src* peptide (*G*), or phospho-EGFR peptide (*H*). Staining was performed by the peroxidase anti-peroxidase method and developed using 3,3'-diaminobenzidine substrate and ethyl green counterstaining. This tumor was an intraductal comedocarcinoma (shown) with some papillary components (not shown).

pression has not been identified in any type of benign breast lesion, including cases of hyperplasia considered to represent possible premalignant lesions. The greatest frequencies of p185 overexpression are observed in ductal carcinoma *in situ* [20–50% overall, and up to 70% of the comedo subtype (16–19, 39)]. The overexpression of p185 at the very earliest stages of mammary carcinoma implies that p185 overexpression initially confers a growth advantage to a malignant clone of cells. While overexpression may be a sufficient stimulus for unregulated proliferation in and of itself, it may also serve to abrogate growth factor or sex hormone dependence or may activate signaling pathways which are synergistic with other concomitant proliferation predisposing alterations. Some evidence suggests that p185 could also play a role in resistance to chemotherapy (63, 64).

The frequency of overexpression drops (to 10–20%) in invasive and metastatic carcinomas. Such a pattern would suggest that p185 may be dispensible at later stages of some of the tumors or that a substantial fraction of invasive carcinomas does not arise from *in situ* precursors. Alternatively, p185 may play a role in later stages of tumor progression, but the protein may be difficult to detect due to down-regulation caused by chronic stimulation by ligands. Finally, changes in autocrine or paracrine agonists may alter the proliferative response to p185 overexpression or even select against overexpression.

Transmodulating agonists for p185 may induce either proliferation (29) or differentiation (65) *in vitro*. Whether the physiological function of p185 is to promote proliferation or differentiation *in vivo* is unknown. It may function in both pathways, depending upon specific spatio-temporal circumstances, either during development or in the mature breast. The ability of p185 to form heterodimers with three other receptors may also alter cross-phosphorylation sites and hence substrate selection. These factors may explain some of the inconsistencies in the clinical data regarding the prognostic value of overexpression. For example, overexpression in the absence of ligand may elicit a proliferative signal and hence a poor prognosis, whereas

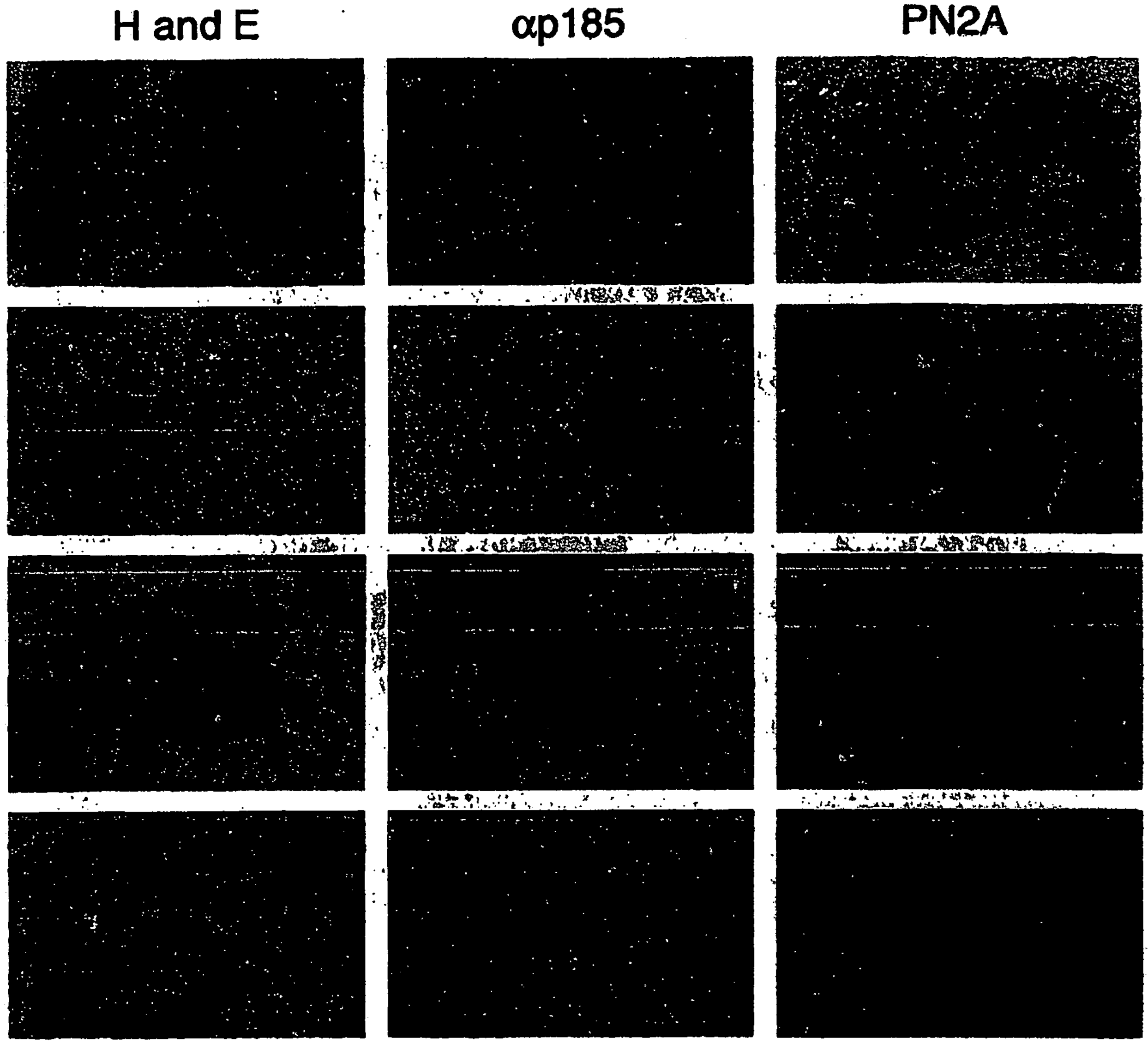

Fig. 6. PN2A immunohistochemistry. Immunohistochemical staining of human breast tumors with anti-p185 Ab-3 (*middle panels*) and PN2A (*right panels*). H & E staining is also shown (*left panels*). The three panels in each horizontal row represent a given tumor. Staining was performed as in Fig. 5. The histologies of these tumors were: *A-C*, micropapillary carcinoma; *D-F*, ductal carcinoma *in situ*, solid variant (shown) with invasive component present elsewhere (not shown); *G-I*, papillary ductal carcinoma *in situ*; *J-L* solid variant (shown) in a comedocarcinoma with associated invasion (not shown).

overexpression in the presence of ligand may induce tumor cells toward terminal differentiation and thus improved prognosis. This paradigm could also explain the observation that p185 overexpression occurs in a much higher proportion of *in situ* malignancies than invasive ones. For example, if a differentiation-inducing ligand produced in the stroma were inaccessible to intraductal carcinoma cells, then p185 overexpression could drive proliferation during the *in situ* phase but then elicit differentiation upon invasion of stroma, at which point negative selection pressure for p185 expression would prevail. Overexpression of receptor tyrosine kinases paradoxically causes the cognate growth factor to elicit growth inhibition even in the absence of induction of differentiation (66–68).

PN2A is an analytical tool that should facilitate the search for answers to many of these questions. It bypasses the need to individually evaluate each of the hormonal inputs in order to predict signaling activity and, by marking the anatomic locations at which p185 is normally active, will help elucidate the source of the most important agonists and the time at which they act. Of the five major autophosphorylation sites in p185, the specific phosphopeptide recognized by PN2A is known to be significant for transformation (Refs. 69 and 70; but see Ref. 71). Some data suggest that this site is the sole autophosphorylation site responsible for oncogenicity as well as coupling to the ras/MAP kinase signaling pathway (72).

On a biological level, correlation of the clinicopathological characteristics of tumors with PN2A staining may illuminate the role of p185 in the biology of these tumors. Correlation with expression of the known EGFR family agonists will demonstrate which of these may be involved in transmodulation of p185 *in vivo*. PN2A can likewise be used to elucidate the biology of p185 during development, menstrual cycle-induced breast changes, and lactation. This general

strategy is applicable to analyses of other phosphoproteins and the role of tyrosine protein kinases in other malignancies. A p185 activation assay utilizing PN2A may also be useful in screening for a p185 activating ligand.

Understanding the precise role of p185 and its agonists is critically important because of the great potential of p185 as a diagnostic marker and because it is a major rational therapeutic target. Improved prognostic accuracy is urgently needed to better guide decisions regarding appropriate adjuvant treatment. We have demonstrated that the extent of p185 tyrosine phosphorylation varies considerably in a small panel of paraffin-embedded sections of p185-overexpressing tumors. Similar data have been obtained on a much larger sample of frozen sections of mammary carcinomas analyzed using our original polyclonal antibody.[7] It is highly likely that measurement of p185 signaling activity as opposed to abundance will greatly enhance the applications of this marker for prognosis and treatment decisions. The first phase II therapeutic trials based upon passive immunotherapy with anti-p185 antibodies will be completed soon (73). In these trials, patients with p185 overexpressing breast tumors are being treated with humanized anti-p185 mAbs which have been selected for their ability to inhibit the growth of cultured cells overexpressing p185 (73). While any p185 overexpressing tumor could theoretically respond to such treatment, the tumors most vulnerable might be those which are dependent upon p185 signaling for growth. If so, PN2A may be uniquely suitable for predicting responses to anti-p185 immunotherapy. These studies represent the vanguard of a series of therapeutic strategies which target p185 (6).

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